

# Cell and plastid division are coordinated through the prereplication factor AtCDT1

Cécile Raynaud<sup>†‡</sup>, Claudette Perennes<sup>†</sup>, Christophe Reuzeau<sup>§</sup>, Olivier Catrice<sup>¶</sup>, Spencer Brown<sup>¶</sup>, and Catherine Bergounioux<sup>†</sup>

<sup>†</sup>Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8618, Bâtiment 630, Université Paris XI, 91405 Orsay, France; <sup>§</sup>Crop Design NV, TechnologiePark3, B-9052 Ghent, Belgium; and <sup>¶</sup>Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette, France

Edited by Marc C. E. Van Montagu, Ghent University, Ghent, Belgium, and approved April 18, 2005 (received for review March 29, 2005)

The cell division cycle involves nuclear and cytoplasmic events, namely organelle multiplication and distribution between the daughter cells. Until now, plastid and plant cell division have been considered as independent processes because they can be uncoupled. Here, down-regulation of AtCDT1a and AtCDT1b, members of the prereplication complex, is shown to alter both nuclear DNA replication and plastid division in *Arabidopsis thaliana*. These data constitute molecular evidence for relationships between the cell-cycle and plastid division. Moreover, the severe developmental defects observed in AtCDT1-RNA interference (RNAi) plants underline the importance of coordinated cell and organelle division for plant growth and morphogenesis.

ARC6 | cell cycle | S phase

Plant morphogenesis results from the combination of cell division and cell differentiation. Accordingly, cell-cycle regulation is an important feature of plant development (1). Reports have focused on the importance of nuclear events such as DNA replication and mitosis. Yet, cell division also involves division and distribution of organelles such as plastids, and the links between cell cycle and plastid division have not been elucidated. Plastids are essential in the viability of plants: many essential genes seem to be involved either in chloroplast biogenesis or chloroplast functions (2). Because plastids cannot be produced *de novo* but originate from other plastids by binary fission, plastid division would be expected to be essential to plant survival, just as it is indispensable to maintain plastid number in dividing cells.

Many studies have highlighted the complexity of mechanisms of plastid division (reviewed in ref. 3), but little is known concerning its regulation. It is considered to be independent of chloroplast differentiation, and some authors also believe it to be independent of cell division because each process can be altered without impairing the other (3). For example, overexpression of plastid division proteins usually results in plastid division inhibition, but the overexpressing plants have no obvious phenotype as far as development or cell division are concerned (4, 5). Conversely, the *arc* (accumulation and replication of chloroplasts) mutants do not show any cell division defects (6). Reciprocally, inhibiting cell division does not necessarily affect plastid division, as observed in cyclin-dependent kinase (CDK) inhibitor NtKIS1a overexpressers: in these plants, the correlation between cell area and chloroplast number is maintained although cell division (but not growth) is dramatically inhibited (7). Overall, these data show that cell division and plastid division can be uncoupled.

Nevertheless, other results suggest that plastid division and cell division are regulated by common pathways. First, the expression of the key plastid division proteins FtsZ seems to be cell cycle-regulated in BY-2 cell suspensions (8) and is induced in developing lateral roots (9). These two facts are consistent with the idea that plastid division must keep pace with cell division so that plastid number is maintained in dividing cells. More strikingly, *Arabidopsis*

*crl* mutants have few but enlarged chloroplasts, and display anomalies in cell division orientation, cell differentiation, and overall plant development (10). Finally, FtsZ1 is localized both in chloroplasts and in the cytoplasm in the moss *Physcomitrella patens* (11), where it seems to form rings at the division site in the cytoplasm as well as in the chloroplasts. Even though it is not yet clear whether the same is true in higher plants, it seems likely that some mechanisms coordinate cell and plastid division, possibly by means of proteins involved in both process.

In yeast and animals, the initiation of DNA synthesis is a complex process that requires many proteins, notably CDT1 and CDC6 (12). Subsequent cyclin-dependent kinase (CDK)-dependent Cdt1/DUP degradation is crucial to avoid re-replication (13). Several actors of early S phase have been shown to be conserved in *Arabidopsis* (14, 15). Two homologues of the *CDT1* gene exist in *Arabidopsis* (16). Castellano *et al.* (17) have shown that AtCDT1a interacts with AtCDC6a and that it is a substrate of the CDK-cyclin D complex. Yet, the function of AtCDT1b, was not investigated. Moreover, AtCDT1a and AtCDT1b share a low degree of identity, suggesting that the two sequences might have diverged (16). This result prompted us to further characterize the function of these genes by observing the effect of their silencing on cell cycle and chloroplast division. Here, we show that simultaneous silencing of both genes induces severe developmental defects, increases the rate of endoreduplication, and inhibits plastid division. Our results show that AtCDT1 proteins are involved not only in nuclear DNA replication but also in plastid division, probably by means of an interaction with the plastid division protein ARC6. As a consequence of this dual function, *AtCDT1* genes are crucial for proper plant development.

## Methods

**Transgene Construct.** The 3' portion of *AtCDT1b* mRNA between positions 1520 and 1762 was amplified by PCR with the primers AtCDT1b XhoI pro (5'-CGCTCGAGGACAGTCATCACGAAGGAGGAGC-3') and AtCDT1b EcoRI rev (5'-GGAATTCAGTAGATAAGTGAAATGTCATGTG-3'). This fragment was subsequently cloned between the Sall and EcoRI of pENT2B. RNA interference (RNAi) constructs were obtained in the Gateway-compatible pHellsgate vector. The cDNA fragment is cloned both sides of an intron: the resulting transcript forms a hairpin structure that triggers silencing of the construct and of the endogene. pHellsgate was designed to obtain such a construct in one step by double recombination between pENT2B and pHellsgate. However, this strategy often led to remodeling of the construct, deletion of the intron or of one insert. We therefore used a modified pHellsgate vector in which an XhoI fragment was removed, resulting in the deletion of one of the

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: *arc*, accumulation and replication of chloroplasts; M5, Murashige and Skoog; RNAi, RNA interference; YFP, yellow fluorescent protein.

<sup>†</sup>To whom correspondence should be addressed. E-mail: raynaud@ibp.u-psud.fr.

© 2005 by The National Academy of Sciences of the USA

ccdB genes and the corresponding recombination sites att1 and att2. The *AtCDT1b* fragment was cloned on this side of the intron between the XhoI and EcoRI sites of the vector. Then, using the Gateway technology, we replaced the second ccdB gene on the other side of the intron by the *AtCDT1b* fragment. In the final construct, the orientation of the fragments was as described in ref. 18.

The full-length cDNA encoding AtCDT1a was amplified by PCR, by using the primers AtCDT1a BamHI (5'-CGGATC-CATGAGTACACCAGGCTCTTC-3') and AtCDT1a SalI (5'-CGTCGACCAGTGGAGCCATGTCTTGCTTC-3'). The PCR product was subsequently sequenced and cloned into the pBI/SmGFP. The fragment encoding AtCDT1a-GFP fusion was also transferred into pCW162. The BamHI-SalI AtCDT1a fragment was also introduced in the pUC-SPYCE vector (19).

The full-length cDNA encoding ARC6 was obtained from Arabidopsis Biological Resource Center (20). It was amplified by PCR, by using the primers ARC6 BglII, and ARC6 SalI. The PCR product was subsequently sequenced and cloned into the pUC-SPYNE vector (19).

**Plant Growth and Transformation.** pHellsgate encoding the AtCDT1-RNAi construct was introduced into *Agrobacterium tumefaciens* (GW3). pCW162 encoding the AtCDT1a-GFP fusion was introduced into the HBA105 strain. *Arabidopsis thaliana* plants (ecotype Wassilewskija) were transformed as described (21). The seeds from the T<sub>1</sub> and T<sub>2</sub> generations were selected on 0.5× Murashige and Skoog (MS) medium containing 50 mg/ml kanamycin. Ten-day-old kanamycin-resistant plantlets were transferred to soil in the greenhouse under long day conditions, and their phenotype was analyzed.

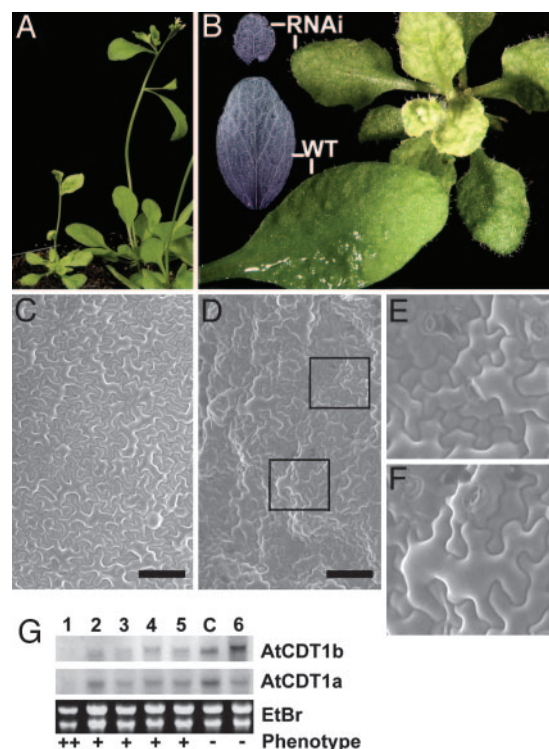
**Transient Expression Assays.** BY-2 cells were cultivated for 2–3 days before protoplast preparation. Protoplasts were obtained by incubating BY-2 cells in an enzymatic solution (1% wt/vol cellulase Onosuka/0.1% wt/vol pectolyase Onosuka, in MS glucose mannitol (MGM) medium) for 30 min at 30°C and 30 min at 37°C. Protoplasts were washed in MGM medium (4.3 g/liter MS/0.17 M mannitol/0.17 M glucose, pH 5.5), and then in MS sucrose (MSS) medium (4.3 g/liter MS/0.28 M sucrose, pH 5.5), counted, and brought to the concentration of 10<sup>6</sup> protoplasts in 150 μl. One million protoplasts were incubated for 15 min at room temperature with 40 μg of each plasmid and 450 μl of polyethylene glycol (PEG) solution (25% wt/vol PEG 6000/0.45 M mannitol/0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>, pH 9). Three milliliters of Ca(NO<sub>3</sub>)<sub>2</sub> 0.275 M were subsequently added to the transformation. Finally, protoplasts were washed in MGM medium and incubated in the dark for 24 h before observation.

**RNA Isolation and Northern Blot Analysis.** RNA extraction and RNA gel blot analysis were performed as described (22). Hybridizations were performed at 62°C in the buffer described by Church and Gilbert (23). The probes for *AtCDT1a* and *AtCDT1b* consist of the full-length cDNA.

**Chloral Hydrate Treatment.** Leaves were fixed in ethanol/acetic acid (9/1 vol/vol) for 1 h, and washed in 90% ethanol and 70% ethanol. Samples were subsequently incubated overnight at room temperature in the dark in chloral hydrate (1 ml of glycerol/2 ml of water/8 g of chloral hydrate).

**Light, SEM, and Confocal Microscopy.** SEM analysis on WT and AtCDT1-RNAi plants was performed as described (1).

Isolated leaf cells from AtCDT1-RNAi plants and WT were prepared as described (5). Cells were stained with 2 μg/ml DAPI dissolved in Galbraith medium supplemented with 1% Triton X-100 (wt/vol), and observed by using an epifluorescence microscope (Zeiss Axioscope). To increase contrast and observe



**Fig. 1.** Silencing of AtCDT1a and AtCDT1b induces severe developmental defects in Arabidopsis. (A) A mature AtCDT1-RNAi plant (left plant) and a WT plant (right plant). (B) Rosette leaves of AtCDT1-RNAi plants (RNAi, top) and WT (WT, bottom); chloral hydrate treatment of the leaves (left) reveals that their vascular tissues are normal. (C–F) SEM of the leaf lower epidermis of WT (C) and AtCDT1-RNAi plant (D–F). (E and F) Shown are images  $\times 4$  the framed areas in D. (Scale bars: 100  $\mu$ m for all panels.) (G) AtCDT1-RNAi plants have reduced levels of both AtCDT1a and AtCDT1b mRNA. This reduction of mRNA abundance correlates with the observed phenotypes. Plants 1, 2, 3, 4, and 5 had the same phenotype as the plant pictured in A and B; plants 6 and 7 were comparable with WT. C, control; EtBr, ethidium bromide.

chloroplasts more easily, starch was stained by macerating the tissues in a solution of lugol (Merck).

Roots of 35S:AtCDT1a-GFP plantlets and transformed protoplasts were observed by using a confocal microscope (Leica TCS, SP2). To observe GFP, excitation was 488 nm, and the spectral detector was set between 510 and 540 nm. To observe yellow fluorescent protein (YFP), excitation was at 514 nm, and the spectral detector was set between 520 and 550 nm.

**Flow Cytometry.** To measure the nuclear DNA content, whole cauline leaves from WT and AtCDT1-RNAi plants were chopped in Galbraith's buffer (24) supplemented with 0.1% Triton X-100 (wt/vol). Extracts were filtered on 48- $\mu$ m-pore nylon, and released nuclei were stained with 2 μg/ml DAPI. Before analysis with the flow cytometer (Elite, Coulter), formaldehyde was added to the samples to 1% (vol/vol). The water-cooled Argon laser (Innova 300, Coherent, Santa Clara, CA) was set in UV at 40 mW.

BrdUrd incorporation was measured in plantlets grown for 2 days on MS medium supplemented with naphthalene-acetic acid as described in ref. 15.

**Chlorophyll and Carotenoid Content.** Two leaf discs with a diameter of 8.5 mm were ground in 750 μl of extraction buffer (50 mM Tris-HCl pH 8/80% wt/vol acetone). Pigments were dosed with a spectrophotometer as described in ref. 25.

**Table 1. Epidermal cell areas in WT and AtCDT1-RNAi leaves**

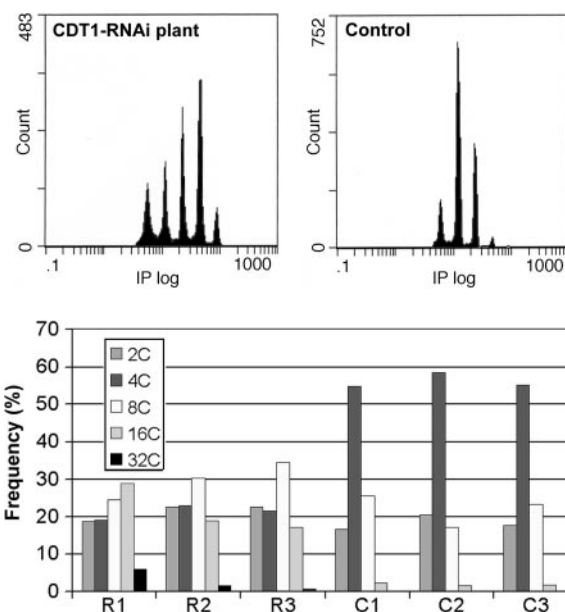
Leaf	Frequency of cell type			Global mean area, ( $\mu\text{m}^2$ )
	<300 $\mu\text{m}^2$	300–3,000 $\mu\text{m}^2$	>3,000 $\mu\text{m}^2$	
WT	0.058 [168]	0.59 [1,407]	0.35 [6,063]	2,957 $\pm$ 234
AtCDT1-RNAi	0.11 [169]	0.79 [1,136]	0.095 [6,027]	1,532 $\pm$ 130

From SEM of leaves, cell areas were measured by using IMAGEJ software. The frequency of cells in each class: area  $\leq 300 \mu\text{m}^2$ , area 300–3,000  $\mu\text{m}^2$ , and area  $\geq 3,000 \mu\text{m}^2$  is reported. The mean cell area ( $\mu\text{m}^2$ ) is indicated in square brackets.

## Results

**Down-Regulation of AtCDT1a and AtCDT1b Results in Severe Developmental Defects.** It has been reported that a single RNAi construct can silence up to three genes with similar sequences (26). To investigate the function of *AtCDT1a* and *AtCDT1b*, we set up RNAi constructs to silence both genes at once. We used a cDNA fragment of *AtCDT1b* chosen in the 3' coding region, well conserved between the two cDNA: they share 62% identity in this region (see Fig. 5, which is published as supporting information on the PNAS web site) versus an overall identity of 52%. Among 96 T<sub>1</sub> kanamycin-resistant plantlets, 5 plants displayed obvious developmental alterations (Fig. 1*A* and *B*). These independent T<sub>1</sub> lines had a reduced stature but showed no delay in flowering time. Their leaves were pale green, crumpled, and smaller than those of WT plants. Nevertheless, chloral hydrate treatment of the leaves revealed that vascular tissues differentiate properly in AtCDT1-RNAi plants because the overall organization of the leaf is comparable with that observed in WT (Fig. 1*B*). The leaves of AtCDT1-RNAi plants observed by SEM had a bumpy surface whereas WT leaves are flat (Fig. 1*C* and *D*). This was due to the distribution of the cells over the leaf surface: the largest cells were clustered on bumps and the smallest ones in hollows. The stomata also seemed to be clustered in hollows rather than homogeneously spread over the whole leaf surface as they are normally. In the WT, projected cell areas range from 60  $\mu\text{m}^2$  to 15,000  $\mu\text{m}^2$  and three classes of cell area can be determined: areas  $\leq 300 \mu\text{m}^2$ , areas 300–3,000  $\mu\text{m}^2$ , and areas  $\geq 3,000 \mu\text{m}^2$ . The minimum and maximum cell areas in CDT1-RNAi plants were the same as in WT, but their frequencies were different. Indeed, these plants simply had more cells in the two smaller categories (Table 1). Surprisingly, the reduction in the average cell area was only 2-fold in AtCDT1-RNAi plants, which is not enough to account for the reduction in size of these plants, suggesting that cell division is also inhibited in AtCDT1-RNAi plants. The reduction of stature of AtCDT1-RNAi plants could thus arise from both cell growth and cell division defects.

To check that these anomalies correlated with *AtCDT1a* and *AtCDT1b* silencing, Northern blot analyses were performed. Indeed, the five T<sub>1</sub> plants previously described had reduced mRNA abundance for both *AtCDT1a* and *AtCDT1b* compared with WT or WT-like T<sub>1</sub> kanamycin-resistant plants (Fig. 1*G*). AtCDT1-RNAi plants accumulate fewer *AtCDT1a* and *AtCDT1b* transcripts than WT, but expression of the two genes is not completely abolished. Therefore, the developmental defects observed seem to be triggered by the simultaneous silencing of *AtCDT1a* and *AtCDT1b*.



**Fig. 2.** AtCDT1-RNAi plants show increased levels of endoreduplication in cauline leaves. (Upper) Flow cytometry analysis of the nuclear DNA content of AtCDT1-RNAi (Upper Left) and control tissue (Upper Right). (Lower) Comparison of the DNA content in nuclei of AtCDT1-RNAi (R1–R3) and control (C1–C3) plants.

**AtCDT1 Silencing Slows Down the Cell Cycle but Increases Endoreduplication.** As stated above, the reduction in cell area was not sufficient to explain the reduction in plant size. This result led us to analyze the rate of cell division in AtCDT1-RNAi plantlets. To this end, BrdUrd incorporation in 15-day-old AtCDT1-RNAi plantlets was measured by flow cytometry. Considering the high rate of endoreduplication in *Arabidopsis*, 4C\* nuclei (\* indicates BrdUrd-positive) can originate from cells that are about to divide, or from cells undergoing endocycles. The division rate can therefore be estimated only by considering nuclei with a 2C\* DNA content, from cells that have undergone a complete cell cycle during the 24-h BrdUrd incubation. The proportion of such nuclei was lower in AtCDT1-RNAi (10.4  $\pm$  0.7%) plantlets than in WT (15.9  $\pm$  1.5%) (Table 2), suggesting that the cell cycle is slowed down in AtCDT1-RNAi plants. Moreover, the overall BrdUrd incorporation was lower in AtCDT1-RNAi plantlets (positive nuclei 24.2  $\pm$  2.2% versus 32  $\pm$  1.8%), suggesting an impairment of S phase. Overexpression of *AtCDT1a* has been reported to increase endoreduplication in rosette leaves (17). This observation prompted us to study the consequences of *AtCDT1* silencing on cell ploidy. WT cauline leaves typically showed 2C to 16C nuclei, with a vast majority of 4C nuclei and only a few 16C nuclei. AtCDT1-RNAi plants showed 32C nuclei and a majority of 8C or 16C nuclei (Fig. 2). Therefore, DNA had replicated once more in AtCDT1-RNAi plants than in WT. This result is all the more striking because it was observed in organs where endoreduplication levels are normally low (27): cauline leaves and young plantlets (data not shown).

**Chloroplast Biogenesis Is Affected in AtCDT1-RNAi Plants.** Sequence analysis of *AtCDT1a* revealed that the protein has a 79-aa-long

**Table 2. Frequency of BrdUrd-positive nuclei in AtCDT1-RNAi plantlets incubated 24 h with 30  $\mu\text{M}$  BrdUrd**

Line	C1	C2	C3	C4	C5	C6	Average	R2	R3	R4	R5	Average
2C*, %	15.6	12.8	13.8	14.9	15.9	22.2	15.8 $\pm$ 1.5	10.5	10.4	12	8.6	10.4 $\pm$ 0.7
BrdUrd-positive, %	30.6	24.8	30.6	33.3	31.9	40.5	32 $\pm$ 1.8	22.1	24.5	30.3	20	24.3 $\pm$ 2.2

C, control plantlets; R, CDT1-RNAi plantlets (100% = total nuclei analyzed).



AtCDT1-RNAi plants, showing that the dramatically enlarged chloroplasts of AtCDT1-RNAi plants contain increased amounts of DNA (see Fig. 6, which is published as supporting information on the PNAS web site). In the WT, the ratio DNA/light scattering is fairly constant. Interestingly, this ratio was also constant in AtCDT1-RNAi plants and for each leaf stage, demonstrating that, even though plastid division is impaired, the correlation between the size of the chloroplast and its DNA content is maintained.

**AtCDT1a Interacts with the Plastid Division ARC6.** To understand how AtCDT1 proteins may participate in plastid division, we tested their interaction with various plastid division proteins in the yeast two-hybrid system. Interestingly, we found an interaction between ARC6 (29) and AtCDT1a (see Fig. 7, which is published as supporting information on the PNAS web site). To confirm this interaction, we tested this interaction in plant cells using the bimolecular fluorescence complementation (BiFC) technique, which allows visualizing protein–protein interactions *in situ* (19, 30). BY-2 protoplasts were cotransformed with two constructs, one encoding a fusion between ARC6 and the N-terminal half of YFP, and the other encoding a fusion between AtCDT1a and the C-terminal half of YFP. As shown previously, no fluorescence was detected after coexpression of the two halves of YFP alone (19, 30). As a control, we transformed plants with a construct encoding a single fusion between a peptide deformylase and GFP that had previously been shown to be targeted to plastids (31). As shown on Fig. 4J–M, GFP and YFP fluorescence accumulated in very similar patterns in BY-2 cells. This result demonstrates simultaneously that AtCDT1a can accumulate in plastids and that it interacts with ARC6 in this cellular compartment, essential for the YFP moieties to reassemble in this imaging strategy.

## Discussion

A moderate reduction in both *AtCDT1a* and *AtCDT1b* mRNA accumulation induces severe and pleiotropic phenotypes. AtCDT1-RNAi plants accumulate fewer *AtCDT1a* and *AtCDT1b* transcripts than WT, but expression of the two genes is not completely abolished. However effective the RNAi strategy may be, the degree of reduction in transcript accumulation has been shown to vary from one target gene to another (32). This fluctuation could be due to variation in the inherent susceptibility of each target to RNAi. It could also be due to the degree of silencing the plant can actually cope with. Lower levels of *AtCDT1a* and *AtCDT1b* transcripts could therefore be lethal. A slight decrease in *AtCDT1a* and *AtCDT1b* transcripts level can, however, be responsible for the severe phenotypes observed. Indeed, a very small increase in CDT1/Dup abundance has been reported to have profound consequences on DNA replication in *Drosophila* (13), suggesting that a very precise quantity of CDT1 protein is required for proper DNA replication. In addition, *Atcdt1b* null mutants have no obvious phenotype (see Fig. 8, which is published as supporting information on the PNAS web site). The defects observed in AtCDT1-RNAi plants are therefore likely to be due to the silencing of both *AtCDT1a* and *AtCDT1b*, although we cannot exclude that *Atcdt1a* null mutants could have the same phenotype.

CDT1 was initially isolated as an essential factor for DNA replication in fission yeast (33). Moreover, CDT1 is essential to DNA replication in human cells (34), in *Caenorhabditis* (35), and in *Drosophila* (36). Our results are consistent with conservation of CDT1 function among eukaryotes. In AtCDT1-RNAi plants, DNA replication is not dramatically inhibited, probably because *AtCDT1* transcript levels are only slightly reduced. This impairment of DNA replication, although moderate, is probably sufficient to increase the required time to complete a cell cycle. Consequently, cells divide less actively in AtCDT1-RNAi plants than in WT plants. Together with the observed reduction of cell size, this inhibition of

cell division could account for the reduced stature of AtCDT1-RNAi plants.

Increased levels of CDT1/DUP have been shown to favor initiation of re-replication in fission yeast (37), *Drosophila* (13), and *Arabidopsis* (17). Considering these results and the essential role of CDT1 in DNA replication, one could expect endoreduplication to be inhibited in AtCDT1-RNAi plants. Surprisingly, silencing of AtCDT1 significantly increased endoreduplication levels more than its overexpression (17). The activity of the prereplication complex proteins can be modulated in a variety of ways (12). Notably, reinitiation of replication is prevented by degradation of CDT1, but also by binding of geminin. The relationships between geminin and CDT1 seem to be complex: geminin inactivates CDT1 protein by binding it, but the levels of their mRNAs are not independent. Indeed, in *Drosophila*, silencing of *geminin* causes marked down-regulation of *Cdt1* (36). This finding highlights the extreme complexity of the regulatory mechanisms underlying the control of DNA replication. The increase of endoreduplication in AtCDT1-RNAi plants could therefore be due to the modification of the abundance of other proteins involved in the control of cell-cycle. For example, we observed by Western blot that the MCM3 protein is more abundant in AtCDT1-RNAi plants than in WT (see Fig. 9, which is published as supporting information on the PNAS web site). Thus, the observed increased in endoreduplication is likely to result from an indirect effect of *AtCDT1a* and *AtCDT1b* silencing. Moreover, the alterations of DNA replication we report here are not a mere consequence of the plastid division defects in CDT1-RNAi plants. Indeed, we measured the DNA content of nuclei in three *arc* mutants (6) with moderate (*arc8*) or severe plastid division defects (*arc10* and *arc6*), and found no alteration of nuclear DNA replication (data not shown).

There is usually a positive correlation between DNA content and cell size in many plant tissues (38). Because endoreduplication levels are increased in AtCDT1-RNAi plants, they could be expected to possess enlarged cells. This hypothesis is at variance with the observation that their cells are in fact smaller than those of WT. Schnitger *et al.* (39), however, have demonstrated that cell growth can be dissected into a DNA-dependent and a DNA-independent expansion program. Their work, together with the results obtained by overexpressing cell cycle inhibitors (1), shows that the relationships between cell size and DNA content are complex. Finally Ramirez-Para *et al.* (40) have shown that an atypical E2F transcription factor can function as a growth regulation factor, contrasting with the well known function of E2F proteins in the control of the G<sub>1</sub>/S transition. This work underlines the complexity of the links between cell cycle and cell growth. Taken together, these studies suggest that endoreduplication is part of a differentiation program in plants. The small size of CDT1-RNAi plant cells regarding their DNA content could be due to a premature onset of this program, forcing small cells to differentiate.

Silencing of *AtCDT1a* and *AtCDT1b* inhibits plastid division in a vast proportion of the cells. This effect was observed on very young plantlets, indicating an inhibition of plastid division *per se* rather than alteration of chloroplast morphology due to early senescence. Several lines of evidence suggest that chloroplast biogenesis is important for leaf development: impairment of chloroplast differentiation often alters mesophyll development through an inhibition of proliferation and/or differentiation of palisade cells (41–44). Other mutants affected in chloroplast biogenesis show more severe developmental alterations that are no longer restricted to mesophyll but affect whole plant morphology (45–47). The crumpled leaf mutant is clear evidence for a relationship between cell division, chloroplast division, and chloroplast differentiation because all three processes are severely affected (10). However, no cell cycle gene affecting chloroplast division and differentiation had been isolated until now. Chloroplast number has been shown to correlate with cell ploidy in plants (48); AtCDT1 might therefore be involved in the

coordination of plastid division with endoreduplication. Nevertheless, a significant proportion of cells in CDT1-RNAi plants contain fewer than 10 chloroplasts. Because meristematic cells contain approximately 10 to 15 proplastids (49), this result indicates that AtCDT1 is also required to coordinate cell and plastid division.

The mechanisms by which AtCDT1 participates in plastid division and differentiation remain to be elucidated. Like a few other plant proteins (50, 51), AtCDT1a can accumulate both in chloroplasts and in the nucleus. Moreover, AtCDT1a interacts with the plastid division protein ARC6. Because ARC6 probably facilitates the polymerization of the plastid division Z-ring (29), AtCDT1 may regulate plastid division by allowing or not the assembly of the plastid division apparatus. This hypothesis is also consistent with the occurrence of plastids of largely different size within the same cell. Such a phenomenon has indeed been reported as a consequence of overexpression of components of the plastid division machinery (5).

Chloroplast DNA content has been suggested to correlate with the size of the organelle (52). The increase of chloroplast DNA content is therefore likely to be an indirect consequence of AtCDT1 down-regulation, and to be due to the increase in chloroplast size. It could also be a way for the plant to cope with the increase in endoreduplication and to set the balance between the nuclear and the chloroplast genome.

Taken together, our results show that plastid division is not independent from cell division. AtCDT1, a member of the prereplication complex, could coordinate plastid and cell division.

We thank Bernard Pineau (Institut de Biotechnologie des Plantes) for helping us with the chlorophyll and carotenoids measures; Séverine Domenichini (Institut de Biotechnologie des Plantes) for assistance with the SEM; Klaus Harter (Zentrum für Molekularbiologie der Pflanzen, Universität Tübingen, Tübingen, Germany) for providing the pUC-SPYNE and pUC-SPYCE vectors; Crisanto Gutierrez (Centro de Biología Molecular Severo Ochoa, Madrid) for providing the AtCDT1a-pGBT-9 construct; Paolo Sabelli (University of Arizona, Tucson, AZ) and Carmella Giglione (Institut des Sciences du Végétal) for providing the PDF:GFP construct; Michael Hodges (Institut de Biotechnologie des Plantes) for providing the anti-ROA and anti-IDH antibodies; Peter Waterhouse (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) for providing the pHellsgate vector; the Arabidopsis Biological Resource Center, the SSP consortium (Salk Institute Genomic Analysis Laboratory in San Diego, the Stanford DNA Sequence and Technology Center in Stanford, CA, and the Plant Gene Expression Center in Albany, CA), Sakis Theologis, and Joe Ecker for providing the ARC6 cDNA; and Roland Boyer (Institut de Biotechnologie des Plantes) for assistance with the figures. We are especially grateful to Rebecca Stevens (Institut National de la Recherche Agronomique, Avignon, France), Francis-André Wollman (Institut de Biologie Physico-Chimique, Paris), Yves Henry (Institut de Biotechnologie des Plantes), and Nathalie Glab (Institut de Biotechnologie des Plantes) for critical reading of the manuscript. The IFR87 confocal microscopy unit is supported by Action de Soutien à la Technologie et la Recherche en Essonne, Conseil de l'Essonne. This work was supported by the Action Concertée Interdisciplinaire (Dynamique et Réactivité des Assemblages Biologiques) from Ministère de l'Éducation Nationale et de la Recherche.

- Jasinski, S., Riou-Khamlichi, C., Roche, O., Perennes, C., Bergounioux, C. & Glab, N. (2002) *J. Cell Sci.* **115**, 973–982.
- Maréchal, E. (2002) *Trends Plant Sci.* **7**, 99–100.
- Osteryoung, K. W. & Nunnari, J. (2003) *Science* **302**, 1698–1704.
- Osteryoung, K. W., Stokes, K. D., Rutherford, S. M., Percival, A. L. & Lee, W. Y. (1998) *Plant Cell* **10**, 1991–2004.
- Raynaud, C., Cassier-Chauvat, C., Perennes, C. & Bergounioux, C. (2004) *Plant Cell* **16**, 1801–1811.
- Pyke, K. & Leech, R. (1992) *Plant Physiol.* **99**, 1005–1008.
- Jasinski, S., Leite, C. S., Domenichini, S., Stevens, R., Raynaud, C., Perennes, C., Bergounioux, C. & Glab, N. (2003) *Plant Physiol. Biochem.* **41**, 667–676.
- El-Shami, M., El-Kafafi, S., Falconet, D. & Lerbs-Mache, S. (2002) *Mol. Genet. Genomics* **267**, 254–261.
- Himanen, K., Vuylsteke, M., Vanneste, S., Vercruysse, S., Boucheron, E., Alard, P., Chriqui, D., Montagu, M., Inzé, D. & Beeckman, T. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 5146–5151.
- Asano, T., Yoshioka, Y., Kurei, S., Sakamoto, W., Sodmergen & Machida, Y. (2004) *Plant J.* **38**, 448–459.
- Kiessling, J., Martin, A., Gremillon, L., Rensing, S. A., Nick, P., Sarnighausen, E., Decker, E. L. & Reski, R. (2004) *EMBO Rep.* **9**, 889–894.
- DePamphilis, M. L. (2003) *Gene* **310**, 1–15.
- Thomer, M., May, N. R., Aggarwal, B. D., Kwok, G. & Calvi, B. R. (2004) *Development (Cambridge, U.K.)* **131**, 4807–4818.
- Stevens, R., Mariconti, L., Rossignol, P., Perennes, C., Cella, R. & Bergounioux, C. (2002) *J. Biol. Chem.* **277**, 32978–32984.
- Stevens, R., Grelon, M., Vezon, D., Oh, J., Meyer, P., Perennes, C., Domenichini, S. & Bergounioux, C. (2004) *Plant Cell* **16**, 99–113.
- Masuda, H. P., Ramos, G. B. A., de Almeida-Engler, J., Cabral, J. M., Coqueiro, V. M., Macrini, C. M. T., Ferreira, P. C. G. & Hemery, A. S. (2004) *FEBS Lett.* **574**, 192–202.
- Castellano, M., Boniotti, M. B., Caro, E., Schnittger, A. & Gutierrez, C. (2004) *Plant Cell* **16**, 2380–2393.
- Siaud, N., Dray, E., Gy, I., Gérard, E., Takvorian, N. & Doutriaux, M. P. (2004) *EMBO J.* **23**, 1392–1401.
- Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevec, D., Grefen, C., Schumacher, K., Oecking, C., et al. (2004) *Plant J.* **40**, 428–438.
- Yamada, K., Lim J., Dale J. M., Chen H., Shinn P., Palm C. J., Southwick A. M., Wu H. C., Kim C., Nguyen M., et al. (2003) *Science* **302**, 842–846.
- Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16**, 735–743.
- Perennes, C., Glab, N., Guglieni, B., Doutriaux, M., Phan, T., Planchais, S. & Bergounioux, C. (1999) *J. Cell Sci.* **112**, 1181–1190.
- Church, G. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
- Galbraith, D. W., Harkins, K. R., Maddox, J. M., Ayres, N. M., Sharma, D. P. & Firoozabady, E. (1983) *Science* **220**, 1049–1051.
- Lichtenthaler, H. (1987) *Methods Enzymol.* **148**, 350–382.
- Miyoshi, K., Ito, Y., Serizawa, A. & Kurata, N. (2003) *Plant J.* **36**, 532–540.
- Bergounioux, C., Brown, S. C. & Petit, P. X. (1992) *Physiol. Plant.* **85**, 374–386.
- Rowan, B. A., Oldenburg, D. J. & Bendich, A. J. (2004) *Curr. Genet.* **46**, 176–181.
- Vitha, S., Froelich, J. E., Koksharova, O., Pyke, K. A., van Erp, H. & Osteryoung, K. W. (2003) *Plant Cell* **15**, 1918–1933.
- Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S. & Ohad, N. (2004) *Plant J.* **40**, 419–427.
- Giglione, C., Serero, A., Pierre, M., Boisson, B. & Meinel, T. (2000) *EMBO J.* **19**, 5916–5929.
- Kerschen, A., Napoli, C. A., Jorgensen, R. A. & Müller, A. (2004) *FEBS Lett.* **566**, 223–228.
- Hofmann, J. F. X. & Beach, D. (1994) *EMBO J.* **13**, 425–434.
- Rialland, M., Sola, F. & Santocanale, C. (2001) *J. Cell Sci.* **115**, 1435–1440.
- Zhong, W., Feng, H., Santiago, F. E. & Kipreos, E. (2003) *Nature* **423**, 885–889.
- Mihaylov, I. S., Kondo, T., Jones, L., Ryzhikov, S., Tanaka, J., Zheng, J., Higa, L. A., Minamino, N., Cooley, L. & Zhang, H. (2002) *Mol. Cell. Biol.* **22**, 1868–1880.
- Yanow, S. K., Lygerou, Z. & Nurse, P. (2001) *EMBO J.* **20**, 4648–4656.
- Kondorosi, E., Roudier, F. & Gendreau E. (2000) *Curr. Opin. Plant Biol.* **3**, 488–492.
- Schnittger, A., Weinl, C., Bouyer, D., Schöbinger, U. & Hülskamp, M. (2003) *Plant Cell* **15**, 303–315.
- Ramirez-Para, E., Lopez-Matas, M. A., Fründt, C. & Gutierrez, C. (2004) *Plant Cell* **16**, 2350–2363.
- Keddie, J. S., Carroll, B., Jones, J. D. G. & Gruissem, W. (1996) *EMBO J.* **15**, 4208–4217.
- Park, S. H., Park, S. H., Chin, H. G., Cho, M. J., Martienssen, R. A. & Han, C. (1999) *Genes Dev.* **14**, 1005–1016.
- Aluru, M. R., Bae, H., Wu, D. & Rodermeier, S. R. (2001) *Plant Physiol.* **127**, 67–77.
- Næsted, H., Holm, A., Jenkins, T., Nielsen, H. B., Harris, C. A., Beale, M. H., Andersen, M., Mant, A., Schneller, H., Camara, B., et al. (2004) *J. Cell Sci.* **117**, 4807–4818.
- Chatterjee, M., Sparvoli, S., Edmunds, C., Garosi, P., Findlay, K. & Martin, C. (1996) *EMBO J.* **15**, 4194–4207.
- Wang, Y., Duby, G., Purnelle, B. & Boutry, M. (2000) *Plant Cell* **12**, 2129–2142.
- Mandel, M. A., Feldmann, K. A., Herrera-Estrella, L., Rocha-Sosa, M. & Leon, P. (1996) *Plant J.* **9**, 649–658.
- Butterfass, T. (1973) *Protoplasma* **76**, 167–195.
- Marrison, J. L., Rutherford, S. M., Robertson, E. J., Lister, C., Dean, C. & Leech, R. M. (1999) *Plant J.* **18**, 651–662.
- Lagrange T., Hakimi M. A., Pontier D., Courtois F., Alcaraz J. P., Grunwald D., Lam E. & Lerbs-Mache S. (2003) *Mol. Cell. Biol.* **23**, 3274–3286.
- Ma, Z. & Dooner, H. K. (2004) *Plant J.* **37**, 92–103.
- Kowallik, K. V. & Herrmann, R. G. (1972) *J. Cell Sci.* **11**, 357–377.